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ORIGINAL ARTICLE

Effects of thymol and diphenyliodonium chloride against *Campylobacter* spp. during pure and mixed culture *in vitro*

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Abstract

Aims: To determine if the purported deaminase inhibitors diphenyliodonium chloride (DIC) and thymol reduce the growth and survivability of *Campylobacter*.
Methods and Results: Growth rates of *Campylobacter jejuni* and *Camp. coli* were reduced compared to unsupplemented controls during culture in Muellar–Hinton broth supplemented with 0·25 μmol DIC or thymol ml^{-1} but not with 0·01 μmol monensin ml^{-1} or 1% ethanol. Recovery of *Camp. jejuni* and *Camp. coli* was reduced >5 $\log_{10}$ CFU from controls after 24 h pure culture in Bolton broth supplemented with 0·25 or 1·0 μmol DIC ml^{-1} or with 1·0 μmol thymol ml^{-1}. Similarly, each test *Campylobacter* strain was reduced >3 $\log_{10}$ CFU from controls after 24 h mixed culture with porcine faecal microbes in Bolton broth supplemented with 0·25 or 1·0 μmol DIC ml^{-1} or with 1·0 μmol thymol ml^{-1}. Treatments with 0·25 μmol thymol ml^{-1}, 0·01 μmol monensin ml^{-1} or 1% ethanol were less effective. Ammonia production during culture or incubation of cell lysates was reduced by 0·25 or 1·0 μmol DIC ml^{-1} but only intermittently reduced, if at all, by the other treatments.
Conclusions: Diphenyliodonium chloride and thymol reduced growth, survivability and ammonia production of *Camp. jejuni* and *Camp. coli*.
Significance and Impact of the Study: Results suggest a potential physiological characteristic that may be exploited to develop interventions.

Introduction

Campylobacter spp. are a leading cause of bacterial associated human foodborne illness in the United States, causing >1·9 million illnesses annually (Mead *et al.* 1999). *Campylobacter* can also cause postinfection complications associated with acquiring immune-mediated neuropathies such as Guillian Barré Syndrome or Miller Fisher Syndrome (Salloway *et al.* 1996; Nachamkin *et al.* 1998). Most *Campylobacter* infections are caused by *Campylobacter jejuni*; however, *Campylobacter coli* recently accounted for more than 25 000 *Campylobacter* infections in the United Kingdom (Tam *et al.* 2003). *Campylobacter* are also recognized as reservoirs of antimicrobial resistance genes that potentially can be exchanged between other pathogenic and commensal bacteria (Engberg *et al.* 2001; Anderson *et al.* 2003; Thakur and Gebreyes 2005; Thakur *et al.* 2006).

Campylobacter can readily colonize the digestive tracts of food-producing animals as evidenced by reports of prevalence exceeding 80% in poultry, swine and beef (Corry and Atabay 2001; Stern *et al.* 2001; Sahin *et al.* 2002; Pearce *et al.* 2003; Stanley and Jones 2003). Poultry and cattle are primarily colonized with *Camp. jejuni* whereas swine are predominantly colonized by *Camp. coli* (Stanley and Jones 2003). However, considerable numbers of pigs (as high as 76%) can be colonized with *Camp. jejuni* (Harvey *et al.* 1999; Jensen *et al.* 2006). Hygienic and biosecurity measures are often employed to control the spread of *Campylobacter* on farms but these frequently are not able to prevent infections (Pattison 2001). Experimental preparations of bacteriocins or bacteriophage have

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proven promising in controlled studies, causing several log-fold reductions in *Campylobacter* concentrations in the gut of turkey poults or broiler chicks (Loc Carrillo *et al.* 2005; Stern *et al.* 2005, 2006; Wagenaar *et al.* 2005; Cole *et al.* 2006). More recently, caprylic acid supplementation to broiler chicks resulted in log-fold reductions in cecal *Camp. jejuni* concentrations (Solis de los Santos *et al.* 2008). At present, however, none of these technologies are commercially available for application in the field.

Campylobacter differ from many other gut microbes in that they lack 6-phosphofructokinase, a key enzyme involved in energy metabolism (Velayudhan and Kelly 2002). As a consequence, *Campylobacter* lack the ability to conserve energy for growth and maintenance via fermentation of carbohydrates. *Campylobacter* can conserve energy via respiration, oxidizing hydrogen and formate for the reduction of electron acceptors such as fumarate, nitrate, sulfites and if at low concentrations, oxygen, to generate proton motive force for electron transport phosphorylization (Laanbroek *et al.* 1978b; Hoffman and Goodman 1982; Smith *et al.* 1999; Sellars *et al.* 2002; Lee and Newell 2006). However, concentrations of these electron acceptors typically are low within gut environments and it is unclear how much growth may be supported by this process. *Campylobacter* can conserve energy via substrate-level phosphorylation during catabolism of the amino acids aspartate, alanine, glutamate, glutamine, methionine and serine (Westfall *et al.* 1986; Mohammed *et al.* 2004; Velayudhan *et al.* 2004). Intermediate and end products of amino acid catabolism by *Campylobacter* include acetate, formate, fumarate, lactate, pyruvate and succinate thus indicating the involvement of mixed acid fermentation and reductive metabolism of the tricarboxcylic acid pathway (Laanbroek *et al.* 1978a; Mendz and Hazell 1996). Velayudhan and colleagues recognized that inhibiting amino-acid catabolism may be a potential target to reduce the competitiveness of *Camp. jejuni* and subsequently showed that a mutant lacking L-serine dehydratase activity, and thus deficient in L-serine catabolism, was unable to colonize the avian gut (Velayudhan *et al.* 2004). Presently, we report results from studies testing the effects of the purported inhibitors of amino-acid catabolism, diphenyliodonium chloride and thymol (Broderick and Balthrop 1979), on *Camp. jejuni* and *Camp. coli* during pure and mixed culture with porcine gut bacteria *in vitro*.

Materials and methods

Campylobacter sources

Campylobacter jejuni used in the present study was a field isolate recovered from poultry (Byrd *et al.* 2001); the *Camp. coli* used was a field isolate recovered from a dairy cow (Callaway *et al.* 2005). Both strains had been preserved in CryoCare™ Bacterial Preservers (Key Scientific Products, Round Rock, TX, USA) according to manufacturer's instructions. Each isolate was resuscitated for each experiment via initial 24 h culture (39°C) in nonantibiotic supplemented Bolton broth (Oxoid Ltd, Basingstoke, Hampshire, UK) and subsequent plating to Campy Cefex agar prepared and used as described by Stern *et al.* (1992). Isolated colonies propagated after 48 h incubation at 42°C were picked and inoculated into Bolton broth supplemented with 0·33 μg cefoperazone and 200 μg cycloheximide per ml which after 24 h incubation (39°C) served as the stock culture for experiments described below.

Pure culture studies

Campylobacter jejuni and *Camp. coli* cultures were inoculated (0·1 ml) via syringe and needle into 18 × 150 mm closed crimp-top culture tubes containing 10 ml Bolton (Oxoid Ltd, Basingstoke, Hampshire, UK) or Muellar–Hinton broth (Becton Dickinson Microbiology Systems, Sparks, MD, USA). The two different media were used to exclude the possibility that observed effects may have been specific to only one medium. Because conditions within the gut ecosystem are anaerobic, cultures were incubated in Bolton broth prepared by boiling and cooling under 100% N_2 gas phase, with the exception that the addition of 5% (v/v) laked horse blood (Lampire® Biological Laboratories, Pipersville, PA, USA) was made after the medium had cooled. Reducing agents were not added to the medium. In preliminary studies, this medium supported an increase of 1·8 ± 0·2 and 1·4 ± 0·3 $\log_{10}$ colony forming units (CFU) ml^{-1} for *Camp. jejuni* and *Camp. coli*, respectively, after 24 h incubation. Cultures incubated in Muellar–Hinton broth were incubated under a microaerobic (N_2 : CO_2 : O_2; 85 : 10 : 5) gas phase as an N_2 atmosphere did not support appreciable growth of either *Campylobacter* in this medium. In all experiments, test compounds were added as small volumes (0·1 ml) of water, ethanol, or concentrated stock solutions of thymol, monensin (each dissolved in ethanol) or diphenyliodonium chloride in water to achieve indicated concentrations in a final volume of 10·1 ml. Thymol, monensin and diphenyliodonium chloride were purchased from Sigma-Aldrich (St Louis, MO, USA). Cultures were incubated upright without agitation at 39°C to approximate in these and the mixed cultures described below the average body temperature of a pig. For cultures grown in Muellar–Hinton broth, optical densities (OD) were measured as absorbance at 600 nm on a Spectronic 20D+ spectrophotometer (Spectronic Instruments, Inc., Rochester, NY, USA) at the times indicated.

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For cultures grown in Bolton broth, fluid samples collected after 0, 6 and 24 h from cultures were serially diluted (10-fold) in 0·1 mol l^{-1} sodium phosphate buffer (pH 6·5) and spread to Campy-Cefex agar. Colonies were counted after 48 h microaerobic ($N_2 : CO_2 : O_2$; 85 : 10 : 5) incubation at 42°C. Fluid samples collected at 0 and 48 h incubation from cultures grown in Muellar–Hinton broth were analysed colorimetrically for determination of ammonia (Chaney and Marbach 1962).

Mixed culture studies

In two separate experiments conducted on separate days, freshly voided porcine faeces obtained from the same donor pig were inoculated (0·2% wet weight/vol) within 30 min of collection into 250 or 300 ml Bolton broth prepared anaerobically as previously described under 100% N_2 gas. These porcine faecal suspensions were then inoculated with 1·0 or 0·6 ml (first and second experiment, respectively) of a 24 h old stock culture of *Camp. jejuni* or *Camp. coli* to achieve approximately 10^6 CFU ml^{-1}. Each faecal suspension was then mixed and dispensed (10 ml volumes) under a continuous stream of N_2 into 18 × 150 mm crimp-top culture tubes preloaded with small volumes (≤0·1 ml) of test compounds described above. Tubes were closed and incubated upright without agitation at 39°C. Fluid samples collected at 0, 6 and 24 h were analysed colorimetrically for determination of ammonia and serially diluted and plated to Campy Cefex agar to enumerate *Campylobacter* as previously described. Endogenous *Campylobacter* in faecal inocula were determined similarly and found to be present at $6{\cdot}0 \times 10^4$ and $1{\cdot}1 \times 10^3$ CFU g^{-1} wet weight (first and second mixed culture experiment, respectively) which corresponds to populations of 120 and 2·2 cells ml^{-1} at the start of each incubation. Total anaerobic bacteria in the faecal inoculum used for the second experiment were enumerated via viable cell count of serial 10-fold dilutions spread to Brucella agar (Anaerobe Systems, Morgan Hill, CA, USA). After 7 days anaerobic ($N_2 : CO_2 : H_2$; 90 : 5 : 5) incubation at 39°C in a Bactron anaerobic chamber (Sheldon Labs Manufacturing Inc., Cornelius, OR, USA), total culturable anaerobes were determined to be present at $5{\cdot}5 \times 10^{10}$ CFU g^{-1} wet weight of faeces which corresponds to a concentration of $1{\cdot}1 \times 10^8$ CFU ml^{-1} upon initiation of incubation.

Cell lysate studies

Cell lysates were prepared from whole cells of *Camp. jejuni* or *Camp. coli* harvested by centrifugation (20 min at 15 000 ***g***) from 24 h old cultures grown in 700 ml anaerobic (100% N_2) Bolton broth. Cells were resuspended in 6 ml phosphate buffer (pH 7·2) and lyzed while chilled on ice by sonication (6 × 30 s at 350 W) using a Braunsonic 1510 sonicator (B. Braun Melsungen AG, San Francisco, CA, USA). Resultant cell lysates were passed into an anaerobic chamber, adjusted to 32 ml via addition of 3 ml 11% casamino acids and phosphate buffer and then used as enzyme addition (1·5 ml) to 2·0 ml microcentrifuge tubes containing additions (≤17 μl) water, ethanol, or concentrated stock solutions of thymol, monensin (each in ethanol) or diphenyliodonium chloride (in water) to achieve a final reaction volume of 1·67 ml. Reactions were conducted at 37°C under anaerobic conditions within the chamber ($N_2 : CO_2 : H_2$; 90 : 5 : 5) and fluid samples (0·4 ml) collected at 0, 3 and 6 h were analysed colorimetrically to determine rates of ammonia production. Protein content of the cell lystates was determined using a commercial Modified Lowry Protein Assay Kit (Pierce, Rockford, IL, USA).

Statistics

All incubations were conducted in triplicate. Mean specific growth rates determined for *Camp. jejuni* and *Camp. coli* after 24 h and final optical density and ammonia accumulations measured after 48 h pure culture in Muellar–Hinton broth were tested for effects of treatment by general analysis of variance. Rates of ammonia accumulation determined during culture in Bolton broth and specific activities of ammonia production determined during incubation of cell lysates were analysed for effects of treatment by general analysis of variance. Log_{10} transformations of CFU ml^{-1} of *Camp. jejuni* and *Camp. coli* during mixed and pure culture experiments in Bolton broth were tested for treatment effects by a repeated measures analysis of variance. Means ($n = 3$) were separated using a LSD comparison of means procedure and differences were considered significant at $P \leq 0{\cdot}05$. All analyses were performed using Statistix®8 Analytical Software (Tallahassee, FL, USA).

Results

Growth curves during pure culture of *Camp. jejuni* and *Camp coli* in Muellar–Hinton broth are presented in Fig. 1. When mean specific growth rates were calculated after 24 h of growth we found that growth rates of *Camp. jejuni* and *Camp. coli* were reduced ($P < 0{\cdot}05$) from those of controls (0·124 ± 0·002 and 0·119 ± 0·002 h^{-1}, respectively) in cultures supplemented with 0·25 μmol diphenyliodonium chloride ml^{-1} (0·057 ± 0·016 and 0·093 ± 0·004 h^{-1}, respectively) or 0·25 μmol thymol ml^{-1} (0·063 ± 0·014 and 0·103 ± 0·008 h^{-1}, respectively). The mean specific growth rate of *Camp. coli* (0·107 ±

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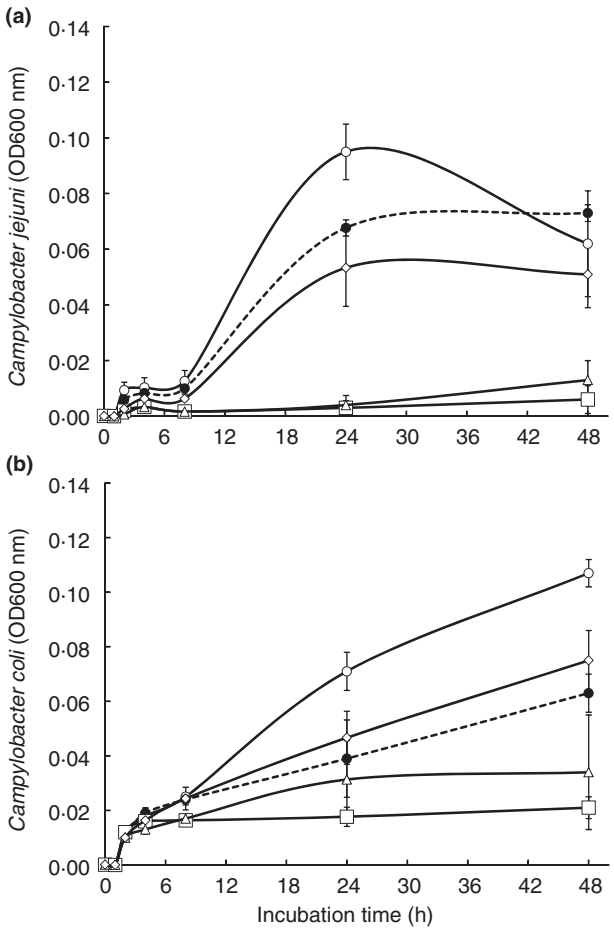

Figure 1 Optical density (OD600 nm) measurements from pure cultures of *Camp. jejuni* (a) or *Camp. coli* (b) incubated microaerobically (N_2 : CO_2 : O_2; 85 : 10 : 5) at 39°C in Muellar–Hinton broth supplemented with water alone (controls, open circles), 1·0% ethanol (closed circles), 0·25 μmol diphenyliodonium chloride ml^{-1} (open squares), 0·25 μmol thymol ml^{-1} (open triangles) or with 0·01 μmol monensin ml^{-1} (open diamonds). Error bars represent standard deviations from n = 3 cultures.

0·008 h^{-1}), but not *Camp. jejuni* (0·118 ± 0·001 h^{-1}) was reduced from that of controls when grown in medium supplemented with 1% ethanol but growth rates were not reduced when grown with 0·01 μmol monensin ml^{-1} (0·111 ± 0·004 and 0·113 ± 0·005 h^{-1}, respectively). An analysis of variance of final optical density measurements made after 48 h growth revealed that by this time *Camp. coli* but not *Camp. jejuni* had achieved less growth in cultures treated with 0·01 μmol monensin ml^{-1} or ethanol than in controls (Fig. 1).

Ammonia accumulations measured after 48 h incubation of *Camp. jejuni* were lower ($P < 0·05$) in cultures supplemented with 0·25 μmol diphenyliodonium chloride or thymol ml^{-1} than in controls or cultures supplemented with ethanol or monensin (Table 1). In the case of *Camp. coli*, ammonia accumulations were reduced from those of controls in cultures supplemented with 0·25 μmol diphenyliodonium chloride or thymol ml^{-1} and to a lesser extent in cultures supplemented with ethanol or monensin (Table 1).

Table 1 Ammonia accumulation (μmol ml^{-1}) after 48 h microaerobic (N_2 : CO_2 : O_2; 85 : 10 : 5) incubation (39°C) of pure cultures of *Campylobacter* or *Campylobacter coli* in Mueller–Hinton broth supplemented with water alone (controls), ethanol, diphenyliodonium chloride, thymol or monensin

Supplement	*Campylobacter jejuni*	*Campylobacter coli*
Water	1·76 ± 0·27^a	1·29 ± 0·26^a
1·0% Ethanol	1·99 ± 0·50^a	0·65 ± 0·50^b
0·25 μmol Diphenyliodonium chloride ml^{-1}	0·12 ± 0·14^b	0·02 ± 0·03^c
0·25 μmol Thymol ml^{-1}	0·07 ± 0·13^b	0·01 ± 0·01^c
0·01 μmol Monensin ml^{-1}	1·68 ± 0·16^a	0·29 ± 0·23^{bc}

Means (±SD) from n = 3 cultures. Values within column with unlike letter superscripts differ ($P < 0·05$).

For pure cultures grown in Bolton broth, treatment with either 0·25 or 1·0 μmol diphenyliodonium chloride ml^{-1} was equally effective in reducing ($P < 0·05$) recovery of *Camp. jejuni* and *Camp. coli* (Fig. 2). Thymol treatment resulted in dose dependent reductions ($P < 0·05$) in the recovery of *Camp. jejuni* or *Camp. coli* compared to the recovery from respective control cultures (Fig. 2), although the inhibitory effect of 0·25 μmol thymol ml^{-1} against *Camp. coli* was slight and did not persist at the 24 h incubation sampling time (Fig. 2b). *Campylobacter jejuni* or *Camp. coli* concentrations in cultures supplemented with ethanol or monensin were not decreased and in some instances were increased during pure culture in Bolton broth (Fig. 2).

Specific activities of ammonia production were reduced ($P < 0·05$) compared to controls by 43–87% in reactions of cell lysates of *Camp. jejuni* and *Camp. coli* supplemented with either 0·25 or 1·0 μmol diphenyliodonium chloride ml^{-1} and by 18–20% by *Camp. coli* lysates supplemented with 0·25 μmol thymol ml^{-1} or with ethanol (Table 2). Specific activities of ammonia production by cell lysates of *Camp. jejuni* and *Camp. coli* supplemented with 1·0 μmol thymol or 0·01 μmol monensin ml^{-1} did not differ ($P > 0·05$) from controls but were increased approx. 51% and 48% by lysates of *Camp. jejuni* supplemented with ethanol or 0·25 μmol thymol ml^{-1} (Table 2).

Recovery of *Camp. jejuni* and *Camp. coli* was reduced ($P < 0·05$) from that in controls during culture with mixed populations of porcine faecal bacteria in Bolton broth supplemented with thymol or diphenyliodonium

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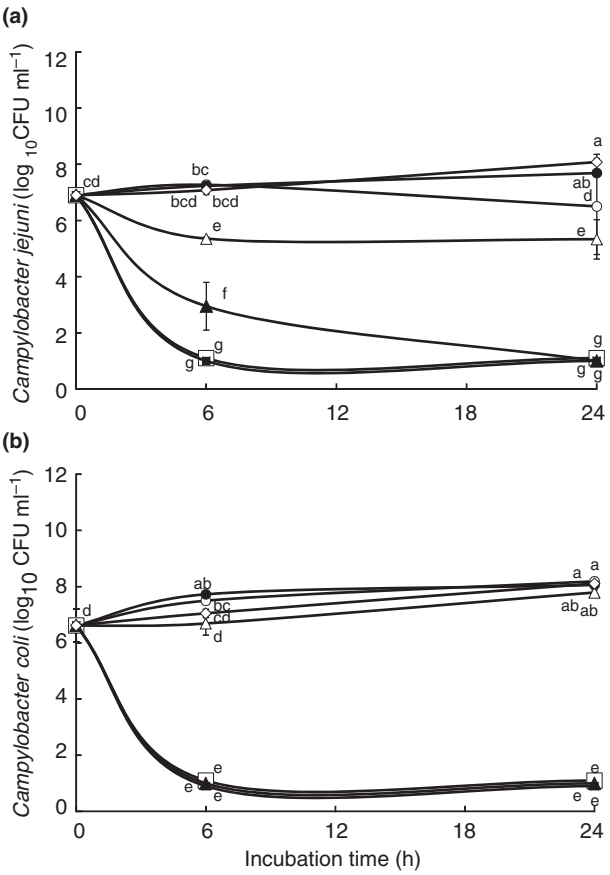

Figure 2 Viable cells counts of *Camp. jejuni* (a) or *Camp. coli* (b) from pure cultures incubated anaerobically (N_2) at 39°C in Bolton broth supplemented with water alone (controls, open circles), 1·0% ethanol (closed circles), 0·25 or 1·0 μmol diphenyliodonium chloride ml^{-1} (open and closed squares, respectively), 0·25 or 1·0 μmol thymol ml^{-1} (open and closed triangles, respectively) or with 0·01 μmol monensin ml^{-1} (open diamonds). Error bars represent standard deviations from n = 3 cultures; values with unlike letter designations differ (P < 0·05).

chloride (Fig. 3). Again, thymol exhibited a dose dependent effect, with supplementation of 1·0 μmol ml^{-1} causing greater reductions than 0·25 μmol ml^{-1}. As observed with the pure cultures, diphenyliodonium chloride was near equally effective at both concentrations (Fig· 3). During mixed culture, recovery of *Camp. jejuni* was reduced from that of controls (P < 0·05) after 24 h in ethanol and monensin-supplemented incubations and the recovery of *Camp. coli* was reduced (P < 0·05) from that of controls in monensin but not ethanol-supplemented incubations (Fig. 3).

Amounts of ammonia produced were higher (P < 0·05) during mixed culture of *Camp. coli* than *Camp. jejuni* with porcine faecal microbes in Bolton broth (Fig. 4) than during pure culture in Muellar–Hinton broth

Table 2 Specific activity of ammonia production (nmol mg prot.$^{-1}$ h^{-1}) during anaerobic (N_2 : CO_2 : H_2; 90 : 5 : 5) incubation (37°C) of cell lysates of *Campylobacter jejuni* or *Campylobacter coli* in phosphate buffer (pH 7·2) supplemented 1·0% casamino acids and with water alone (controls), ethanol, diphenyliodonium chloride, thymol or monensin

Supplement	*Campylobacter jejuni*	*Campylobacter coli*
Water	90·33 ± 29·94^{c}	75·51 ± 4·14^{ab}
1·0% Ethanol	136·22 ± 37·70^{a}	62·21 ± 3·11^{c}
0·25 μmol Diphenyliodonium chloride ml^{-1}	51·23 ± 3·60^{de}	9·65 ± 4·12^{d}
1·00 μmol Diphenyliodonium chloride ml^{-1}	29·90 ± 2·67^{e}	17·89 ± 6·75^{d}
0·25 μmol Thymol ml^{-1}	133·60 ± 4·86^{ab}	60·62 ± 4·77^{c}
1·00 μmol Thymol ml^{-1}	103·62 ± 4·02^{bc}	65·62 ± 10·31^{bc}
0·01 μmol Monensin ml^{-1}	69·31 ± 3·93^{cd}	79·63 ± 5·24^{a}

Means (±SD) from n = 3 cultures. Values within column with unlike letter superscripts differ (P < 0·05).

(Table 1). Consistent with the suspected inhibition of deaminase activity, rates of ammonia accumulation during culture of mixed populations of porcine faecal bacteria inoculated with *Camp. jejuni* or *Camp. coli* were decreased (P < 0·05) from that of nontreated controls (0·328 ± 0·01 and 0·861 ± 0·01 μmol NH_3 ml^{-1} h^{-1}, respectively) in incubations treated with 0·25 μmol ml^{-1} (0·062 ± 0·01 and 0·092 ± 0·02 μmol NH_3 ml^{-1} h^{-1}, respectively) and 1·0 μmol diphenyliodonium chloride ml^{-1} (0·035 ± 0·01 and 0·027 ± 0·01 μmol NH_3 ml^{-1} h^{-1}, respectively) (Fig. 4). Rates of ammonia accumulation were also decreased from that of controls (P < 0·05), albeit to a lesser extent, in incubations supplemented with monensin (0·272 ± 0·01 and 0·396 ± 0·04, μmol NH_3 ml^{-1} h^{-1} respectively) or 1·0 μmol thymol ml^{-1} (0·108 ± 0·01 and 0·593 ± 0·08 μmol NH_3 ml^{-1} h^{-1}, respectively). Rates of ammonia accumulation during mixed cultures with *Camp. coli* were decreased from controls (P < 0·05) with 0·25 μmol thymol ml^{-1} but did not differ (P > 0·05) with ethanol (0·731 ± 0·02 and 0·848 ± 0·02 μmol NH_3 ml^{-1} h^{-1}, respectively). Conversely, rates of ammonia accumulation during mixed culture with *Camp. jejuni* were increased marginally (P < 0·05), when treated with 0·25 μmol thymol ml^{-1} or ethanol (0·385 ± 0·03 and 0·439 ± 0·02 μmol NH_3 ml^{-1} h^{-1}, respectively) (Fig. 4).

Discussion

Results from the present study demonstrate that diphenyliodonium chloride is a potent inhibitor of *Camp. jejuni* and *Camp. coli* both in pure or mixed culture. When tested at 0·25 and 1·0 μmol ml^{-1}, diphenyliodonium chloride was equally inhibitory to *Camp. jejuni* and *Camp.*

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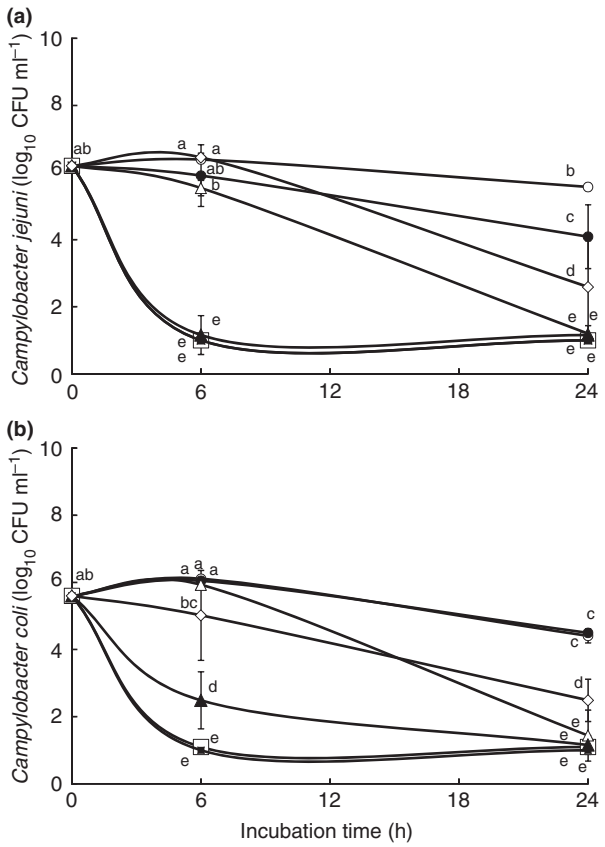

Figure 3 Viable cells counts of *Camp. jejuni* (a) or *Camp. coli* (b) from mixed cultures of porcine faecal bacteria incubated anaerobically (N_2) at 39°C in Bolton broth supplemented with water alone (controls, open circles), 1·0% ethanol (closed circles), 0·25 or 1·0 μmol diphenyliodonium chloride ml^{-1} (open and closed squares, respectively), 0·25 or 1·0 μmol thymol ml^{-1} (open and closed triangles, respectively) or with 0·01 μmol monensin ml^{-1} (open diamonds). Error bars represent standard deviations from n = 3 cultures; values with unlike letter designations differ (P < 0·05).

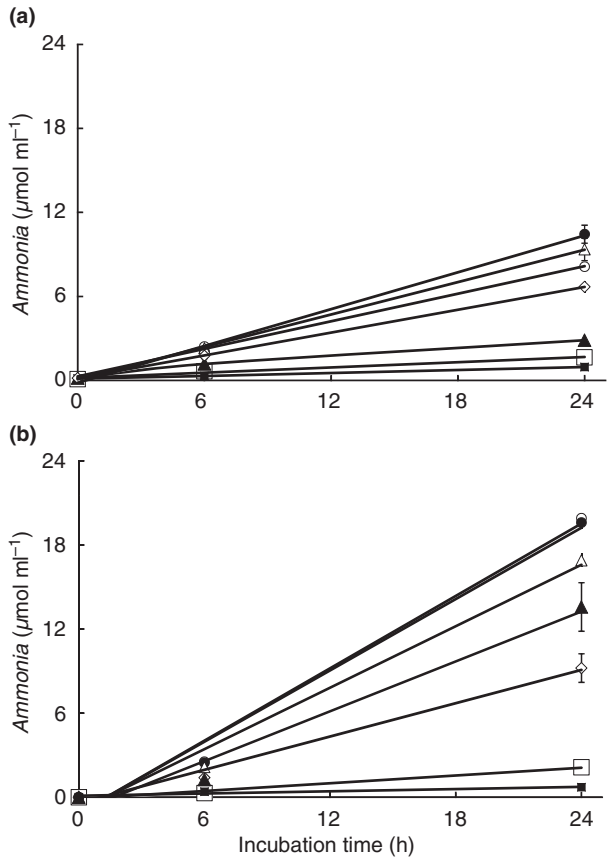

Figure 4 Rates of ammonia accumulation from mixed cultures of porcine faecal bacteria inoculated with *Camp. jejuni* (a) or *Camp. coli* (b) and incubated anaerobically (N_2) at 39°C in Bolton broth supplemented with water alone (controls, open circles), 1·0% ethanol (closed circles), 0·25 or 1·0 μmol diphenyliodonium chloride ml^{-1} (open and closed squares, respectively), 0·25 or 1·0 μmol thymol ml^{-1} (open and closed triangles, respectively) or with 0·01 μmol monensin ml^{-1} (open diamonds). Error bars represent standard deviations from n = 3 cultures.

coli indicating that the minimal inhibitory concentration of this compound had not yet been reached (Figs 2 and 3). Others have reported in numerous studies that thymol, as an essential oil or plant extract, is inhibitory to a variety of bacteria, including *Escherichia coli, Pseudomonas, Salmonella* and *Campylobacter* (Helander *et al.* 1998; Friedman *et al.* 2002; Si *et al.* 2006). Results from our study extend these findings, demonstrating that thymol inhibited *Camp. jejuni* and *Camp. coli* during pure culture as well as during mixed culture with porcine gut microbes thus supporting reports that thymol exhibited antimicrobial activity in swine intestinal contents (Michiels *et al.* 2007) and animal wastes (Varel and Miller 2000, 2001, 2004; Varel 2002; Varel *et al.* 2006, 2007; Varel and Wells 2007). Contrary to that observed with diphenyliodonium chloride, thymol was more effective against both *Campylobacter* species when supplemented at 1·0 than at 0·25 μmol ml^{-1} to our Bolton broth incubations (Figs 3 and 4). For comparison, the minimum bactericidal concentrations of thymol against *E. coli* K88, *E. coli* O157:H7 and *Salm.* Typhimurium DT104 were 0·66, 1·10 and 1·55 μmol ml^{-1}, respectively (Si *et al.* 2006). The minimum effective concentration of thymol against total anaerobes within the pig small intestine was found to be 1·72 μmol ml^{-1} and was 2·66 μmol ml^{-1} against coliforms (Michiels *et al.* 2007). *Campylobacter* concentrations from cultures treated with 1·0 μmol thymol ml^{-1} and both levels of diphenyliodonium chloride were at times reduced to our limit of detection, which was 10 cells ml^{-1}. This indicates that *Campylobacter* endogenous to the faecal inocula, estimated

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to be present at 120 or 2·2 cells ml^{-1} at the start of each incubation (in the cultures in Fig. 3a,b, respectively) had also been reduced or prevented from achieving appreciable growth in these particular cultures.

Other than their membrane-disruption properties (Helander *et al.* 1998; Burt 2004; Oussalah *et al.* 2006; Varel and Wells 2007), little has been reported regarding other potential mechanistic effects of thymol against pathogenic bacteria in general and specifically against *Campylobacter*. Diphenyliodonium chloride reacts with flavin and flavoprotein moeities (Chakraborty and Massey 2002) and inhibits NADPH oxidase activity (Moulton *et al.* 2000). Thus, diphenyliodonium chloride could potentially inhibit oxidation/reduction reactions involved in amino acid catabolism. Ammonia accumulation during both pure (Table 1) and mixed culture (Fig. 4) was nearly abolished with diphenyliodonium chloride and intermediately reduced with thymol. These results from our culture studies do not allow us to distinguish whether or not our observed decrease in ammonia production was due to inhibition of deaminase activity or to the presence of fewer viable amino acid-catabolizing cells in the affected cultures. However, specific activities of ammonia production by cell lysates of *Camp. jejuni* and *Camp. coli* were inhibited by both diphenyliodonium chloride treatments which support our hypothesis that these concentrations inhibited amino acid catabolizing-activity in these crude enzyme preparations (Table 2). Results from reactions of cell lysates with thymol were less clear as specific activities of ammonia production were marginally reduced by lysates of *Camp. coli* treated with 0·25 μmol thymol ml^{-1} and were increased by similarly-treated lysates of *Camp. jejuni* lysates (Table 2). Specific activities by lysates of *Camp. jejuni* and *Camp. coli* treated with 1·0 μmol thymol ml^{-1} did not differ from controls (Table 2). In contrast to the cell lysate reactions, rates of ammonia accumulations were not reduced by ethanol during mixed culture with either *Camp. jejuni* or *Camp. coli* compared to nontreated controls (Fig. 4) although the final accumulation of ammonia was reduced by *Camp. coli* during pure culture (Table 1). Specific activities of ammonia production by cell lysates of *Camp. jejuni* and *Camp. coli* were not inhibited by monensin (Table 2), thus indicating that the monensin-caused inhibition of ammonia production observed during pure and mixed culture was likely due to inhibition of bacterial growth and not due to inhibition of amino-acid catabolism *per se*.

Monensin is inhibitory to a number of Gram-positive ammonia-producing bacteria from rumen or swine waste environments (Callaway *et al.* 2003; Cotta *et al.* 2003), some of which conserve energy exclusively via amino acid fermentation. In pure culture, growth rates of *Camp. jejuni* or *Camp. coli* (Fig. 1) or viable cell recovery (Fig. 2) were significantly reduced by monensin, however, comparisons of the final optical density measurements made at 48 h revealed that monensin and ethanol treatments exhibited modest inhibition ($P < 0·05$) of *Camp. coli* but not *Camp. jejuni*. When grown in mixed culture, ethanol reduced the recovery of *Camp. jejuni* and monensin reduced the recovery of both *Camp. jejuni* and *Camp. coli*, albeit to a lesser extent than that observed with thymol or diphenyliodonium chloride (Fig. 3). These observations suggest that the *Campylobacter* were less able to resist the effects of ethanol and monensin in the competitive environment. Our observation that *Campylobacter* were not markedly inhibited by monensin, at least during the first 24 h of growth, is not unexpected, as Gram-negative bacteria are typically less sensitive to this ionophore antibiotic than Gram-positive bacteria (Callaway *et al.* 2003).

During mixed culture of porcine faecal bacteria with inoculated *Campylobacter*, amounts of ammonia produced in unsupplemented controls (Fig. 3) were much higher than during pure culture of unsupplemented *Campylobacter* controls (Table 1). This likely reflects the contribution of bacteria other than *Campylobacter* within the mixed cultures to the production of ammonia as *Campylobacter* were determined in the second experiment to be present at <1% the total population of anaerobes. Thus, the inhibition of ammonia production during mixed culture by both diphenyliodonium chloride treatments and the 1·0 μmol thymol ml^{-1} treatment likely reflects inhibition of amino acid and peptide catabolism primarily by bacteria other than *Campylobacter*. However, inhibition of amino acid catabolism by *Campylobacter* cannot be excluded. Both diphenyliodonium chloride and thymol, or an essential oil product containing thymol, have been studied in ruminants for their ability to reduce the energetically wasteful process of ruminal protein degradation (Broderick and Balthrop 1979; Chalupa *et al.* 1983a,b; Newbold *et al.* 2004) which implicates their potential ability to inhibit amino acid catabolism, and thus disrupt energy conservation, as a potential mode of action against *Campylobacter*.

Varel and Miller (2000) recognized the deaminase inhibiting potential of diphenyliodonium chloride for control of gaseous emissions from livestock waste and Velayudhan *et al.* (2004) reported that a *Camp. jejuni* mutant lacking L-serine dehydratase activity, and thus deficient in L-serine catabolism, was unable to colonize the avian gut. Our results suggest that inhibition of amino acid metabolism with chemical inhibitors may be a metabolic target for the control of foodborne *Campylobacter*. Whether or not diphenyliodonium chloride, thymol or other chemical inhibitors could possibly be

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synthesized or modified, will undoubtedly depend on further assessments of their efficacy and potential toxicity *in vivo*. Precedence exists for the application of thymol and diphenyliodonium chloride to animals. For instance, diphenyliodonium chloride and an essential oil product containing thymol have been administered to ruminants, as inhibitors of the energetically wasteful process of ruminal protein degradation, with no reported adverse effects in short term studies (Chalupa *et al.* 1983a,b; Newbold *et al.* 2004). Additionally, essential oil products containing thymol have been administered to pigs with no reported adverse effects on digestibility or animal performance (Muhl and Liebert 2007a,b) and have improved digestibility in poultry (Hernández *et al.* 2004). Thymol has also been shown to effectively inhibit ammonia production from added L-leucine by ruminal microbes without causing detrimental effect to the mixed population even after 37 days of semi-continuous culture (Ramirez Piñeres *et al.* 1997). If products were developed that preferentially delivered the inhibitors to the lower gastrointestinal tract, inhibition of amino acid fermentation may be nutritionally advantageous if they were to protect amino acids from degradation and thus increase their availability for absorption in the small intestine. Moreover, inhibition of amino acid degradation may decrease the production of potentially toxic end products, such as ammonia, amines (i.e. histamine, putrescine, cadaverine, piperidine, and others), volatile phenols and indoles (Smith and Macfarlane 1997; Hughes *et al.* 2000; Apajalahti 2005). From a regulatory viewpoint, thymol is a natural plant product and as such may be viewed favourably as an alternative to antibiotics (Wallace 2004). Moreover, barring the assimilation of unacceptable residues, it may be feasible to administer either diphenyliodonium chloride or thymol as a terminally applied feed additive, given a few days before harvesting to reduce *Campylobacter* colonization and transmission during transport and at slaughter. Such an application may be particularly beneficial for poultry and swine, as transportation from the farm to the processing plant may contribute to high concentrations of *Campylobacter* entering the plant (Stern *et al.* 1995; Harvey *et al.* 2001). Conversely, transportation from feedlot to the abattoir had little effect on faecal shedding of *Campylobacter* in feedlot cattle (64% and 68%, respectively) and significantly reduced hide contamination (Beach *et al.* 2002). However, *Campylobacter* prevalence is reported to increase during the feeding period, from 1·6% near or upon entry to the feedlot to 63% approximately four months later (Besser *et al.* 2005). Thus, strategic feeding of inhibitors of amino acid catabolism may help reduce the transmission of *Campylobacter* throughout cattle in the feedlot while concurrently reducing the digestive inefficiency associated with ruminal protein degradation. Whereas monensin is commonly fed to feedlot cattle, purportedly to inhibit ruminal amino acid metabolism by hyper ammonia-producing anaerobes such as *Clostridium aminophilum*, *Cl. sticklandii* and *Peptostreptococcus anaerobius* (Callaway *et al.* 2003), our results reveal that *Campylobacter* are not particularly sensitive to this ionophore. Whether monensin would negatively affect *Campylobacter* colonization in bovine gut, or perhaps more importantly, exert a more severe inhibition of bacteria that may compete against campylobacteria for amino acid substrates is unknown. If the latter event were to occur, it may inadvertently promote the ability of *Campylobacter* to grow and colonize the lower bovine gastrointestinal tract. This hypothesis, however, has yet to be tested and previous attempts to link an effect of monensin feeding to increased carriage of foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* have not been substantiated (Garber *et al.* 1995; Dargatz *et al.* 1997; Callaway *et al.* 2003; Edrington *et al.* 2003a,b; Van Baale *et al.* 2004).

In summary, results from the present study confirm that diphenyliodonium chloride and thymol markedly reduce the growth and recovery of *Campylobacter* during both pure and mixed culture and suggest a physiological characteristic that may potentially be exploited for the development of interventions.

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